Cell Cycle Changes in the Buoyant Density of Exponential-Phase Cells of Streptococcus faecium

DAVID T. DICKER AND MICHAEL L. HIGGINS*

Department of Microbiology and Immunology, Temple University School of Medicine, Philadelphia, Pennsylvania 19140

Cell buoyant densities were determined by centrifugation in Percoll gradients containing exponential-phase cells of Streptococcus faecium ATCC 9790 grown at a mass doubling time of about 33 min. This bacterium showed the highest average density values (1.13 g/ml) measured to date for any eucaryotic or procaryotic organism. Fractions having the highest densities were enriched with cells that were in the process of dividing or had just divided. These high-density fractions were also enriched with cells that had newly initiated sites of cell wall growth. It appears that S. faecium shows minimum cell densities in the midportion of its cycle.

For some time the buoyant densities of cells have been measured with colloidal suspensions of silica by gradient centrifugation (H. E. Kubitschek, Crit. Rev. Microbiol., in press). Recently, the confidence in such measurements has increased due to (i) the introduction of Percoll (a suspension of colloidal silica coated with polyvinylpyrrolidone [Pharmacia Fine Chemicals, Piscataway, N.J.]), which has a low affinity for cell surfaces (12) and low osmolarity, and (ii) an accumulation of knowledge as to how growth conditions can affect buoyant density measurements (Kubitschek, in press). With these advances, values for the variance in the buoyant density of Escherichia coli during its cell cycle have decreased from an early estimate of ~5%, which was obtained from an analysis of the distribution of cells in Ludox gradients (colloidal silica [19]), to the currently accepted figure of <1%, which was derived from Percoll gradients (12, 15, 24). The current value of <1% suggests that the buoyant density of E. coli changes little during its cell cycle (10, 12, 13). This conclusion recently was reinforced and extended by showing that exponential-phase cells of E. coli fractionated on the basis of size in sucrose gradients yielded invariant buoyant densities (12).

In contrast with E. coli, it appears that the buoyant density of Saccharomyces cerevisiae varies significantly during its cell cycle (1, 6). This confirms the variable densities observed in slide culture-grown cells of Saccharomyces cerevisiae by Mitchison, who used interference light microscopy (17). Mitchison also observed density changes in growing cells of Schizosaccharomyces pombe (16) and Streptococcus faecalis (18). However, the inability of other workers to confirm these findings for Schizosaccharomyces pombe (14) with Percoll has led to criticism of slide culture results (Kubitschek, in press).

In this study we examined the claim of Mitchison that there are density changes in chain-forming streptococci by using Percoll gradients.

MATERIALS AND METHODS

Growth. To analyze accurately cells banded in Percoll equilibrium density gradients, it was necessary to adjust the osmolarity of the Percoll solution. This done by adding concentrated growth medium to stock solutions of Percoll before gradients were made. In the case of Streptococcus

---

* Corresponding author.

---

faecium ATCC 9790, the chemically defined medium (21) normally used to grow the organism cannot be made in a concentration high enough to adjust the final osmolarity of Percoll to the osmolarity of the growth medium.

To overcome this problem, we grew Streptococcus faecium in 20% growth medium and used undiluted growth medium to adjust the osmolarity of Percoll stocks (see below). The mass doubling time determined turbidimetrically (21) was 30 to 33 min.

The cultures analyzed by equilibrium density centrifugation were required to undergo at least 10 exponential-phase mass doublings (determined turbidimetrically [21]). Chilled, exponential-phase cells equivalent to ca. 2 μg of dry mass per ml (21) were used to inoculate tubes containing 20% growth medium and were allowed to undergo six exponential-phase mass doublings. At this time the culture was used to inoculate 10 ml of prewarmed 20% growth medium with sufficient cells to bring the culture to the equivalent of ca. 13 μg (dry mass) per ml. When this culture had gone through at least four mass doublings and reached the equivalent of 215 μg (dry cell mass) per ml, it was harvested for study.

Fractionation of cells by gradient centrifugation. The culture was either chilled in an ice bath or fixed by adding formaldehyde to a final concentration of 0.37% for 30 min at room temperature. This period of fixation was sufficient to inhibit growth and cellular autolysis, as shown by no detectable change in turbidity when fixed cells were incubated for 18 h in 20% growth medium at 37°C. Because of the high buoyant density of Streptococcus faecium cells, it was necessary that stock solutions of Percoll (1.129 g/ml) be concentrated to 1.249 g/ml by packing dialysis tubing containing stock Percoll in sodium carboxy methyl cellulose (Calbiochem-Behring, La Jolla, Calif.) for ca. 5 h at 4°C. The osmolarity of the concentrated Percoll was adjusted with undiluted growth medium by making a solution composed of 67% (vol/vol) concentrated Percoll (1.249 g/ml), 20% undiluted growth medium, and 13% water. This solution was then diluted to form stocks of the desired density with 20% growth medium, which were used in turn to make 35-ml linear gradients (1.096 to 1.134 g/ml) in tubes (25 by 89 mm).

After being chilled or fixed, cells were concentrated by centrifugation (8,000 × g, 10 min, 4°C), and the equivalent of 2.15 mg of dry cellular mass was suspended in 1 ml of the lowest-density Percoll-growth medium stock used to form a gradient. Gradients with cells layered on top were centrifuged for 10 min in a Beckman SW27 swinging bucket rotor
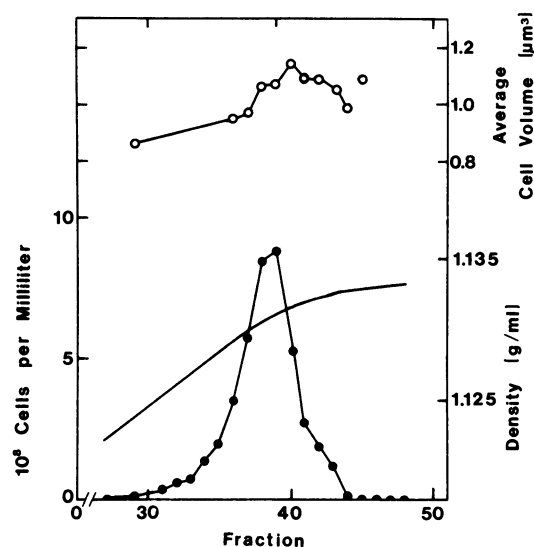
FIG. 1. Average *Streptococcus faecium* cell volume (O) and cell number (●) found in successive fractions of a Percoll gradient. The solid line indicates the average density. Cell numbers and volumes were estimated by electronic particle analysis.

at 22,500 × *g* at 4°C for unfixed cells and at 22°C for fixed cells. Increasing the centrifugation time to 20 min did not result in a change in cell band position or average buoyant density. Also, introducing cells into the gradient during preparation at the average density of exponential-phase cultures of *Streptococcus faecium* (at ca. 1.13 g/ml) resulted in banding of cells at the same buoyant density as observed in cells that were loaded from the top of the gradient. Gradients were collected at 4°C (unfixed cells) or 22°C (fixed cells) from the top of each tube in 1-ml fractions by displacement from the bottom with 65% (wt/wt) sucrose. The numbers of cells and their volumes were measured with an electronic particle counter (Particle Data, Elmhurst, Ill.) from Formalin-fixed samples diluted with 0.9% saline (4). Buoyant densities were estimated from measurements of indices of refraction of fractions with an Abbe refractometer.

Electron microscopy. The methods used for Formalin fixation, critical-point drying, carbon-platinum replica preparation, electron microscopy, and three-dimensional reconstruction applied to electron micrographs of replicas to estimate the volume of cells and their components have been described previously (2, 3). The three-dimensional reconstruction technique consisted of using a digitizer interfaced with a computer (model 4051; Tektronix, Beaverton, Oreg.) to convert the perimeters of cells seen in electron micrographs of replicas into *x* and *y* coordinates (2). The coordinates were mathematically rotated with the computer around the central axis of each cell to estimate the volume of the polar caps and growth sites seen in the cell. The volume estimates and various other linear measurements were stored on magnetic tape in such a way that the measurements for each pole and growth site of each cell in a population could be addressed separately. For this study, at least 100 cells were reconstructed from each gradient fraction analyzed.

RESULTS AND DISCUSSION

Separation of cells by density. Figure 1 shows the distribution of the number of unfixed exponential-phase cells of
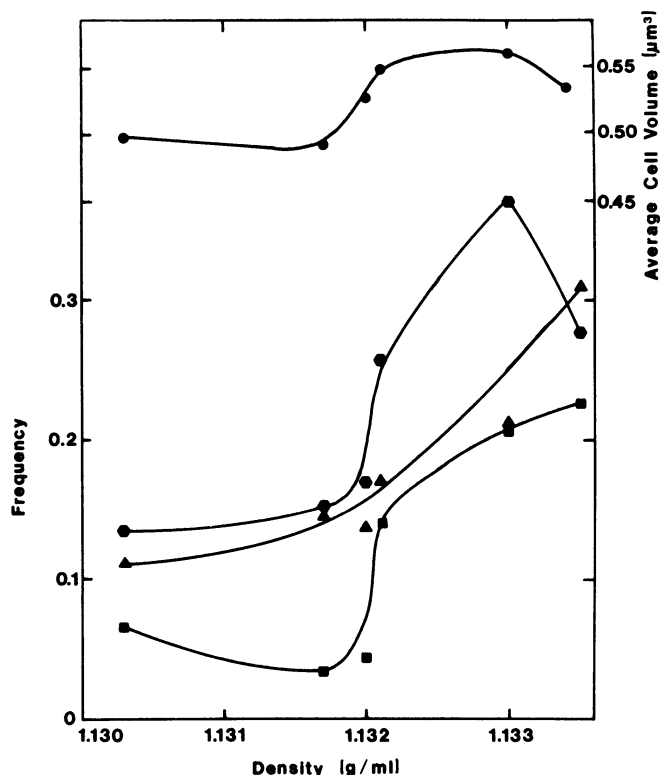

FIG. 2. Average cell volume (●) and frequency of cells with small (>0- but <0.12-μm³) central (▲) and peripheral (■) cell wall growth sites and cells in the division phase (cells with sites which were >0.2488 μm³) (◗), as determined by three-dimensional reconstruction of replicas of cells taken from gradient fractions. Each set of points represents an analysis of at least 100 cells from a fraction.

Streptococcus faecium as a function of buoyant density as measured by Percoll gradient centrifugation. The analysis of such gradients gave an average cell density of 1.1309 g/ml (range, 1.1301 to 1.1312 g/ml), which is greater than the value measured for any procaryotic organism investigated to date. The closest value to the *Streptococcus faecium* value is the 1.114-g/ml estimate reported for the yeast *Saccharomyces cerevisiae* (Kubitschek, in press).

The cells of *Streptococcus faecium* appear to be very dense, and the average coefficient of variation for the density of these cells is 0.25%. This is very close to the value obtained for *E. coli* when similar methods were used (0.15% [12]). Figure 1 shows that, unlike the volume of *E. coli*, the volume of *Streptococcus faecium* determined with an electronic particle counter changed with density. With increasing density the volume of unfixed and fixed cells of *Streptococcus faecium* increased and then decreased slightly in the highest-density fractions. This pattern of change was also seen when cell volumes were measured from three-dimensional reconstructions of electron micrographs of replicas of cells taken from gradient fractions (Fig. 2 and 3). However, it should be noted that due to the dehydration that cells must undergo in preparation for electron microscopy, the volumes of cells calculated from electron micrographs are smaller than those measured in a particle counter. From the frequency distributions of volumes in electron micrographs, we found that the decreases in volume in the highest-density

classes (>1.132 g/ml) were due to increases in the frequency of smaller cells (Fig. 3).

We concluded that the average cell volume changes in *Streptococcus faecium* as a function of density; this finding is dissimilar to results obtained for *E. coli* with comparable methodology but similar to results obtained for *Saccharomyces cerevisiae* (1).

Further examination of the frequency distributions of cell volumes from each fraction showed an increase in cells with small cell wall growth sites with increasing density (Fig. 3). To help explain these observations in terms of growth

Central Growth Site

Peripheral Growth Sites

FIG. 4. Diagrammatic representation of the cell cycle of *Streptococcus faecium*. In the early and mid portion of the cell cycle, the cell grows principally by the enlargement of the central growth site (i.e., the cross-hatched zone). As the central site approaches a maximum size of about 0.25 μm^3 and division, new cell wall growth sites (i.e., the stippled zones) appear at peripheral locations.
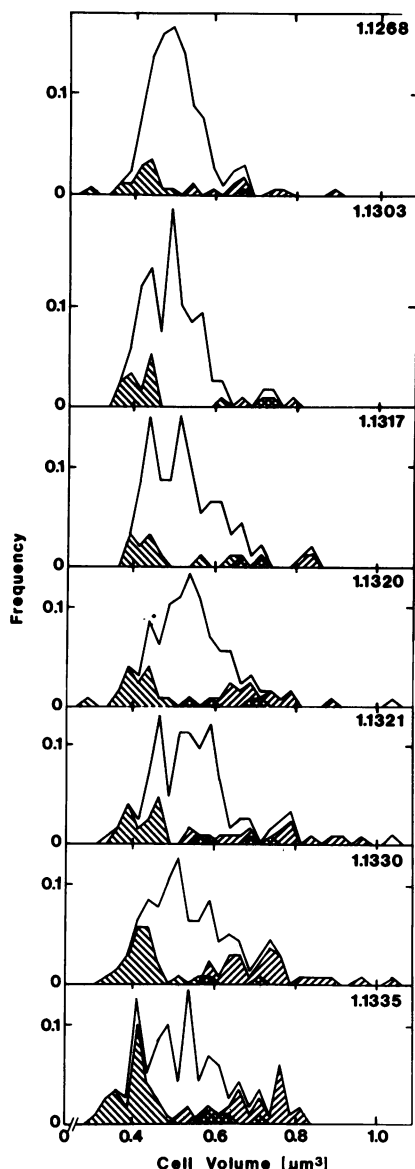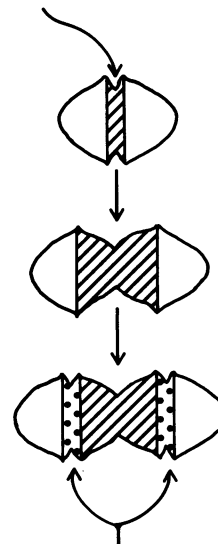

FIG. 3. Distributions of cell volumes in populations of cells reconstructed from electron micrographs of cell replicas, taken from fractions of Percoll gradients as a function of increasing buoyant density. In each population, the distributions of the whole cell volumes (poles plus growth sites) (solid lines) and the distribution volumes of these same cells that had small (>0- but <0.12-μm^3) central (▨) and peripheral (▥) growth sites are shown. The number in the upper right corner of each panel refers to the density of the fraction analyzed (in grams per milliliter).

stages, Fig. 4 shows a diagrammatic representation of a cell cycle of a rapidly growing culture of *Streptococcus faecium*. At the mass doubling times used in these experiments (30 to 33 min), newly divided cells usually have a small cell wall growth site in a central location between the polar caps (3). It has been shown that such a site is capable of growing to a maximum size of ca. 0.25 μm^3 (2, 3). As a site approaches this maximum size, new sites must be introduced in order for the cell to continue to grow in volume. In rapidly growing cultures, these new sites are initiated at peripheral locations on either side of the central site before division. On division, these peripheral sites become the central sites of the newly divided cells. Thus, small newly initiated cell wall growth sites can be observed in both central and peripheral locations.

For purposes of analysis, we defined a small site, regardless of location, as being >0 but <0.12 μm^3. It was by selecting cells with growth sites in this size range from data stored on magnetic tape gathered from the three-dimensional reconstructions of populations of replicas of cells that the distributions of subpopulations of cells with small sites were determined in Fig. 2 and 3.

Figure 2 shows the increase in the frequency of cells in each population with small sites at central and peripheral locations as a function of increasing density. This figure also shows the frequency of cells in the terminal stages of division (i.e., those with central sites whose volume is >0.2488 μm^3). It is clear that with increasing density there was an increase in the frequency of large dividing cells with newly initiated sites at peripheral locations (i.e., cells with central sites that were >0.2488 μm^3 which also had peripheral sites that were >0 but <0.12 μm^3) and small freshly divided cells with newly initiated sites in the central location (i.e., cells with central sites that were >0 but <0.12 μm^3).
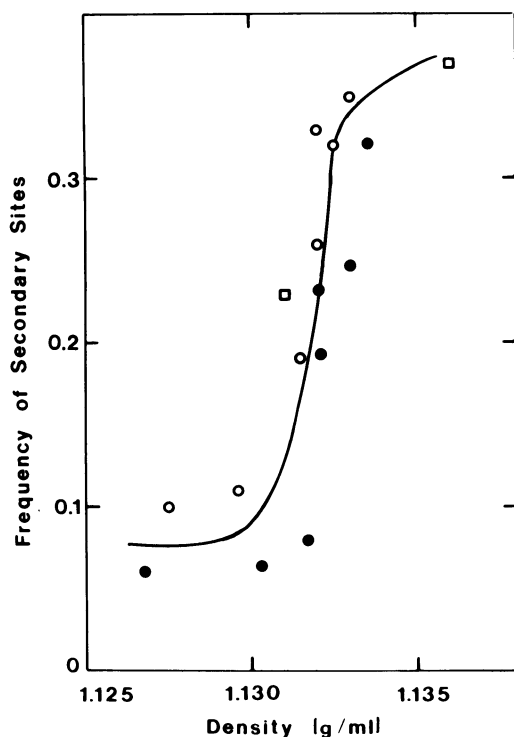
FIG. 5. Distribution of unfixed cells with small peripheral cell wall growth sites as a function of buoyant density. A different symbol was used for each of the experiments analyzed. Each determination represents an analysis of at least 100 cells.

These data indicate that cells with the lowest density are found in the midportion of the cell cycle; the cells with the highest densities are observed just before and after division.

Reproducibility and sources of error. To examine the reproducibility of our findings, we measured the frequency of cells that had small peripheral sites in fractions of several gradients (Fig. 5). We selected the small-peripheral-site morphological characteristic because we could determine the number of cells in a population of replicas with small sites in this location by direct electron microscopic counts without having to go through the laborious procedure of three-dimensional reconstruction. Figure 5 shows that the increase in the frequency of cells with small peripheral growth sites determined by electron microscopic examination of cells found in gradient fractions of increasing density was quite reproducible from gradient to gradient. In each case at densities greater than about 1.13 g/ml there was a sharp increase in the frequency of cells with small peripheral sites.

It was important to explore several sources of error that could affect the results obtained from Percoll gradient centrifugation analysis (Kubitschek, in press). These sources included (i) cells that were not centrifuged long enough to reach equilibrium, (ii) cells that changed in density due to growth or loss of materials during centrifugation or gradient fractionation, and (iii) cells that were affected by osmotic changes that could occur as cells passed through the gradient. In considering these sources of error, we concluded that the 10-min period of centrifugation that we used was sufficient to allow cells to come to equilibrium because we obtained similar average densities (1.130 ± 0.002 g/ml) and comparable increases in the frequency of cells with small

peripheral sites (i.e., an increase from 0.12 to 0.34 as densities increased from below to above 1.13 g/ml) when cells were centrifuged for 20 min and when cells were introduced into gradients during preparation at a level corresponding to 1.13 g/ml. Also, it appeared that our results were not due to growth or autolytic processes, for cells fixed with 0.37% Formalin yielded average densities in a similar range (1.13 g/ml) and similar increases in the frequency of cells with small peripheral sites at higher densities (i.e., an increase from 0.14 to 0.26 as densities increased from below to above 1.13 g/ml) as measured in unfixed cells. Our comparison of the average densities of the fixed and unfixed cells must be qualified in that the gradients used to analyze these cells were centrifuged at 4 and 22°C, respectively; however, the important finding appears to be that the fixed cells showed a distribution of cell cycle stages as a function of density similar to that observed in the unfixed cells. Finally, it seems unlikely that our results were due solely to osmotic effects, for we obtained a separation of cell cycle stages when fixed cells were centrifuged on Percoll gradients to which no additions of medium components had been made (i.e., under these conditions the average density of cells was somewhat lower [1.120 g/ml], but the increase in the frequency of cells with small peripheral sites showed a similar pattern [i.e., an increase in frequency from 0.10 to 0.34 as densities increased from below to above 1.120 g/ml]) similar to the separation seen in unfixed cells analyzed in the presence of growth medium components.

We concluded that cell cycle stages of *Streptococcus faecium* cells can be reproducibly separated on the basis of density and that this separation cannot be related to any source of error which we considered.

Implications of results on models of cell growth. Our data are consistent with the interference microscopy observations of Mitchison (18) of 25 years ago, which showed cell cycle changes in the density of chain-forming streptococci. However, the data of Mitchison are difficult to compare with our data because of the high variability which he observed between density and cell volume. In contrast, our findings indicate a clear reproducible increase in density near the division event. As stated above, in a recent review Kubitschek considered *Saccharomyces cerevisiae* and *Streptococcus faecium* (unpublished data) to be the only examples of organisms that vary significantly in density during their cell cycles (Kubitschek, in press). He suggested that in part this might be due to the fact that cells of *Saccharomyces cerevisiae* and *Streptococcus faecium* have very high density values or large amounts of dense cell wall or both. An additional argument might be that unlike *E. coli*, which shows little variation in density but which rapidly changes its dimensions under different growth conditions (5, 22, 23, 25), *Streptococcus faecium* demonstrates little variation in size in relation to its cell cycle or changes in growth conditions (2–4, 8). Thus, the cell cycle changes in density seen in *Streptococcus faecium* may be due to the relative inability of this organism to quickly change overall cell dimensions. This argument probably applies to *Saccharomyces cerevisiae* as well, in which only small changes in the dimensions of the mother cell occur during the budding process (9). Also, the surface of *Saccharomyces cerevisiae* and the surface of *Streptococcus faecium* grow in fixed increments. The mother cell of *Saccharomyces cerevisiae* (9) and a cell wall growth site of *Streptococcus faecium* (2–4) share the common characteristic that once either the mother cell or the central cell wall growth site reaches a maximum volume, it is incapable of continued rapid increases in size.

Thus, for rapid growth to proceed, the mother cell of *Saccharomyces cerevisiae* must bud, and a cell of *Streptococcus faecium* with a large central envelope growth site must form new cell wall growth sites (Fig. 4).

In view of this pattern of growth, it has been proposed that *Streptococcus faecium* might initiate new sites of cell wall growth in response to an increase in cytoplasmic density or pressure due to the fact that the cytoplasm is growing faster than the surface of cells whose cell wall growth sites have reached their maximum size (11). The hypothesis that density could control surface growth also has been proposed by Rosenberger et al. (20). Certainly the increase in the frequency of *Streptococcus faecium* cells with small sites as a function of increasing density is consistent with such a model. However, the fact that many of the cells with the highest densities contain small growth sites indicates that the appearance of new sites does not likely result in an immediate decrease in density. Some time might be needed before a newly formed cell wall growth site can begin the rapid growth which can lead to a decrease in density.

These interpretations are tentative, for we know little about the physical and biochemical basis of the apparent increases in density which we see in *Streptococcus faecium* near division. For example, a recent autoradiographic study (7) of *Streptococcus faecium* showed, at the 95% confidence level, no change in the concentration of either RNA or protein in exponential-phase cells. Either the measurements were not critical enough to detect the very small changes in density which we measured in our analysis of cells fractionated on Percoll gradients, or the cell cycle changes in density in *Streptococcus faecium* are related to changes in the concentrations of substrates other than RNA or protein.

Additional work will be required before *Streptococcus faecium* buoyant density measurements can be accurately interpreted in relation to the physiology of the organism. This system offers a valid approach for studies of the regulation and synthesis of the cell surface of this organism.

ACKNOWLEDGMENTS

LITERATURE CITED

1. **Baldwin, W. W., and H. E. Kubitschek.** 1984. Buoyant density variation during the cell cycle of *Saccharomyces cerevisiae*. J. Bacteriol. **158:**701–704.
2. **Edelstein, E. M., M. S. Rosenzweig, L. Daneo-Moore, and M. L. Higgins.** 1980. Unit cell hypothesis for *Streptococcus faecalis*. J. Bacteriol. **143:**499–505.
3. **Gibson, C. W., L. Daneo-Moore, and M. L. Higgins.** 1983. Initiation of wall assembly sites in *Streptococcus faecium*. J. Bacteriol. **154:**573–579.
4. **Gibson, C. W., L. Daneo-Moore, and M. L. Higgins.** 1984. Analysis of initiation of sites of cell wall growth in *Streptococcus faecium* during a nutritional shift. J. Bacteriol. **160:**935–942.
5. **Grossman, N., E. Z. Ron, and C. L. Woldringh.** 1982. Changes in cell dimensions during amino acid starvation of *Escherichia coli*. J. Bacteriol. **152:**35–41.
6. **Hartwell, L. H.** 1970. Periodic density fluctuation during the yeast cell cycle and the selection of synchronous cultures. J. Bacteriol. **104:**1280–1285.
7. **Higgins, M. L., A. L. Koch, D. T. Dicker, and L. Daneo-Moore.** 1986. Autoradiographic studies of the synthesis of RNA and protein as a function of cell volume in *Streptococcus faecium*. J. Bacteriol. **167:**960–967.
8. **Higgins, M. L., and G. D. Shockman.** 1976. Study of a cycle of cell wall assembly in *Streptococcus faecalis* by three-dimensional reconstruction of thin sections of cells. J. Bacteriol. **127:**1346–1358.
9. **Johnson, B. F.** 1965. Morphometric analysis of yeast cells: adult cell volume of *Saccharomyces cerevisiae*. Exp. Cell Res. **39:**577–583.
10. **Koch, A. L., and G. Blumberg.** 1976. Distribution of bacteria in the velocity gradient centrifuge. Biophys. J. **16:**389–405.
11. **Koch, A. L., and M. L. Higgins.** 1984. Control of wall band splitting in *Streptococcus faecalis*. J. Gen. Microbiol. **130:**735–745.
12. **Kubitschek, H. E., W. W. Baldwin, and R. Graetzer.** 1983. Buoyant density constancy during the cell cycle of *Escherichia coli*. J. Bacteriol. **155:**1027–1032.
13. **Kubitschek, H. E., W. W. Baldwin, S. J. Schroeter, and R. Graetzer.** 1984. Independence of buoyant cell density and growth rate in *Escherichia coli*. J. Bacteriol. **158:**296–299.
14. **Kubitschek, H. E., and R. A. Ward.** 1985. Buoyant density constancy of *Schizosaccharomyces pombe* cells. J. Bacteriol. **162:**902–904.
15. **Martinez-Salas, E., J. A. Martin, and M. Vincente.** 1981. Relationship of *Escherichia coli* density to growth rate and cell age. J. Bacteriol. **147:**97–100.
16. **Mitchison, J. M.** 1957. The growth of single cells. I. *Schizosaccharomyces pombe*. Exp. Cell Res. **13:**244–262.
17. **Mitchison, J. M.** 1958. The growth of single cells. II. *Saccharomyces cerevisiae*. Exp. Cell Res. **15:**214–221.
18. **Mitchison, J. M.** 1961. The growth of single cells. III. *Streptococcus faecalis*. Exp. Cell Res. **22:**208–225.
19. **Poole, R. K.** 1977. Fluctuation in buoyant density during the cell cycle of *Escherichia coli* K12: significance for the preparation of synchronous cultures by age selection. J. Gen. Microbiol. **98:**177–186.
20. **Rosenberger, R. F., N. B. Grover, A. Zaritsky, and C. L. Woldringh.** 1978. Control of microbial surface-growth by density. Nature (London) **271:**244–245.
21. **Shockman, G. D.** 1962. Amino acids, p. 567–673. *In* F. Kavanaugh (ed.), Analytical microbiology. Academic Press, Inc., New York.
22. **Trueba, F. J., E. A. van Spronsen, J. Traas, and C. L. Woldringh.** 1982. Effects of temperature on the size and shape of *Escherichia coli* cells. Arch. Microbiol. **131:**235–240.
23. **Trueba, F. J., and C. L. Woldringh.** 1980. Changes in cell diameter during the division cycle of *Escherichia coli*. J. Bacteriol. **142:**869–878.
24. **Woldringh, C. L., J. S. Binnerts, and A. Mans.** 1981. Variation in *Escherichia coli* buoyant density measured in Percoll gradients. J. Bacteriol. **148:**58–63.
25. **Woldringh, C. L., N. B. Grover, R. F. Rosenberger, and A. Zaritsky.** 1980. Dimensional rearrangement of rod-shaped bacteria following nutritional shift-up. II. Experiments with *Escherichia coli* B/R. J. Theor. Biol. **86:**441–454.